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Mini-review

Heterogeneity in cancer stem cells

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ABSTRACT

Accumulating evidence suggests that cancer stem cells (CSCs) are heterogeneous populations and their phenotypes are unstable. A number of intrinsic and extrinsic mechanisms contribute to CSC phenotypic variation. The existence of various CSC subpopulations which would lead to a rapid relapse after primary treatments might pose a problem for CSC targeted therapeutics. In order to develop more effective approaches to cancer therapeutics, more CSC-related surface markers or targeting molecules, as well as some novel targeting strategies should be explored. This review summarized the origin and performance of heterogeneity in CSCs and discussed their therapeutic implications.

Introduction

In the past two decades, significant progress has been made in identifying the tumorigenic cancer cells responsible for tumor initiation, maintenance and drug-resistance. This small subset of tumorigenic cancer cells has been termed as cancer stem cells (CSCs) [1,2]. The first prospective identification of CSCs came from studies of acute myeloid leukemia (AML) [3,4], in which leukemic stem cells were defined as $CD34^+CD38^-$ phenotype similar to normal hematopoietic stem cells [4]. Subsequently, CSCs were also isolated and characterized in solid tumors such as breast cancers [5], brain cancers [6], colon cancers [7] and some other tumor types [8–10]. To date, isolation and characterization of CSCs largely depend on surface markers shared with normal stem cells [11]. For example, CD133, which was initially described as a surface marker for human hematopoietic progenitor cells [12], has been widely explored as a reliable marker for CSCs in many tumor types [10,13,14]. Identified by related biomarkers, even in the same tumor mass, there would be CSC subpopulations residing in different fractions [15]. Several studies demonstrated that $CD34^+CD38^-$ was not the only phenotype in leukemic stem cells [16,17]. Similarly, CD166 was reported to be a reliable surface marker for lung CSC enrichment, compared with CD133, CD44 or EpCAM [18], and both $CD271^+$ and $CD271^-$ melanoma cells in NOD/SCID mice could initiate new tumors [19]. The variations ranging from genotype to phenotype of CSCs, which cause heterogeneous in macroscopic feature, microscope appearance and clinical outcomes, define the heterogeneity of CSCs [20]. CSC population is phenotypic heterogeneous among tumor types and even within the same tumor subtype, which are termed as intertumor and intratumor heterogeneity respectively [2,11]. Heterogeneity of CSCs, especially the intratumor heterogeneity, poses the challenge to the targeting therapy in clinic. Herein, we reviewed current progress on heterogeneity in CSCs and discuss therapeutic implications of CSCs.

Sources of heterogeneity in cancer stem cells

The early studies revealed that genetic mutation contributed to tumor initiation and progression [21]. As an inherent factor influencing cell physiological activities, distinct genomic profiles [2] of CSCs originating from different tumors is one of the main explanations for variation of intertumor CSCs. In addition, analogous to an ecosystem, tumors are complicated organizations that individual cells would also interact with the microenvironment where they resided. Accompanying the intrinsic variation among intratumor CSC subclones, microenvironment would be another part of factors that causes intratumor heterogeneity [22,23]. In brief, the heterogeneity of CSCs would be contributed by intrinsic and microenvironmental differences, which share similar explanations with cancer cell heterogeneity [2,24,25]. Intrinsic differences encompass genetic mutations and epigenetic changes, whereas cell–cell interactions, various chemotactic factors, cytokine concentrations and hypoxic conditions are considered to be microenvironmental differences [2,26].

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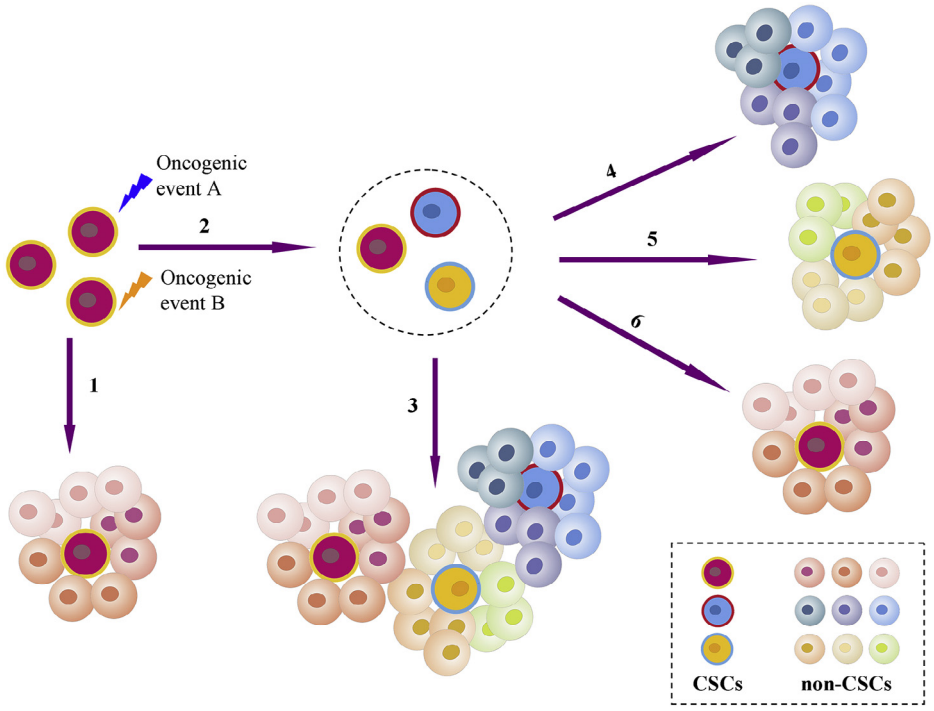

Fig. 1. Schematic illustration of clonal evolution contributing to CSC heterogeneity. It is supposed to have different routes for CSC evolution and differentiation. (1) One initial CSC clone is able to form all populations within a tumor. The CSCs phenotype is homogeneous in this tumor. (2) Different mutations result in multiple CSC subclones. (3) These subclones contribute to tumor growth and CSC heterogeneity exists in an individual tumor. (4–6) More adapting CSC subclones survive under different environmental pressures, which result in CSC heterogeneity among patients or locations.

As one of the intrinsic factors, the instability of CSC genome which includes increased point mutation frequency and chromosomal instability, results in the genetic heterogeneity of CSC [23]. Meanwhile, CSCs undergo clone evolution which brings new subclones [2,25]. These subclones with multiple molecular mechanisms exhibit varying malignant potentials. For example, Emlet and colleagues found that $CD133^+$ glioblastoma stem cells with EGF receptor (EGFR) possessed the highest tumorigenic potential [27]. Then, under different environmental pressures, the more adapting and aggressive CSC subclones with adaptable genomic mutation to the selections will become dominant population and contribute to tumor formation (Fig. 1) [2,25].

The advanced sequencing technology and epigenome research also highlight the crucial role of epigenetic modification in the diversity of CSCs, which is considered to be another intrinsic factor governing CSC heterogeneity. The epigenetic reprogramming in cancer cells involve in epigenetic factors adjustment and DNA methylation, as well as altering the chromatin states through chromatin regulators [21,28]. For example, the single-cell RNA-sequencing demonstrated that five primary glioblastoma samples presented different transcriptone profiles [29]. In melanoma, H3K4 demethylase JARID1B could be used as a biomarker to isolate a subpopulation of slow-cycling melanoma cells [30]. MOZ (monocytic leukemic zinc finger) histone acetyltransferase in AML is associated with poor prognosis [31]. The non-coding RNAs, for instance, the microRNAs (miRNAs), also play crucial roles in epigenetic heterogeneity in CSCs. The miR-451 was upregulated in $CD133^-$ glioblastoma cells, and the stemness of cell would be weakened when transfected with miR-415 [32]. Altogether, the instability of CSC genomes and the variation of epigenetics co-regulate the heterogeneity of CSCs.

Tumor development is not only manipulated by cancer cell intrinsic factors, but also influenced by the extrinsic compartments which are termed as microenvironment or niche. Microenvironmental differences (e.g., hypoxia, acidosis and reactive oxygen species) are selective pressures, as well as inducers of cell genetic instability [33]. Thus, different microenvironments or niches would contribute to CSC phenotypic and functional heterogeneity [25,33]. For instance, in squamous cell carcinomas, two CSC subclones differing in CD34 expression levels could interchange their phenotype according to different microenvironments [34]. In addition, hypoxic and perivascular niches are also vital conditions for the maintenance of CSC properties [35]. Conley et al. showed that hypoxic niche increased the CSC fraction in breast cancer [36]. Similarly, brain and skin cancer stem cells were found in a perivascular niche where autocrine VEGF could sustain CSCs stemness [37,38]. The diversity of some tumor-related extrinsic components, such as cancer-associated fibroblasts (CAFs), tumor vascular network and immune cells also contribute to CSC heterogeneity [21,39]. In these niches, the secreted factors and the activation of related signaling would regulate the conversion between CSCs and non-CSCs. For instance, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) or transforming growth factor-β (TGF-β) secreted from myofibroblast could activate Wnt signaling or Zeb1 promoter transcription, which would induce dedifferentiation of non-CSCs to CSCs [22,40,41]. On the contrary, when the Wnt or Notch signaling was downregulated, miR-27 was upregulated or cells were activated by BMP4, CSCs tend to differentiate to non-CSCs (Fig. 2) [42,43]. All these findings indicated that the heterogeneity of CSCs was the consequences of interaction of intrinsic and extrinsic factors.

Diversity of CSC phenotype

Benefiting from the advent of fluorescence-activated cell sorting (FACS) technology, it is possible to sort phenotypically distinct subpopulations to investigate their functional potential *in vivo*. Using this approach, a number of markers have been found useful for

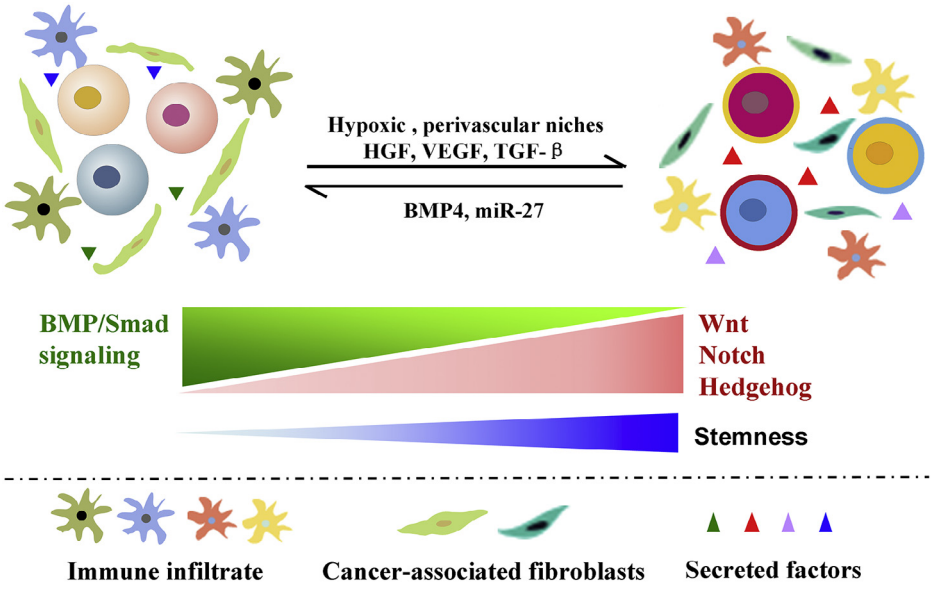

Fig. 2. Cancer cells exhibit plasticity driven by different microenvironments.

defining CSCs in multiple types of tumors (Table 1). Moreover, CSCs also have been defined as subpopulations sharing functional features with normal stem cells, such as side population (SP) [70], sphere cells [10] and ALDH1$^+$ cells [71].

In line with heterogeneous populations, the CSC phenotype may not be universal among patients [25]. Singh et al. had firstly reported that CD133 which could be identified by AC133 antibodies was a robust marker for brain CSCs [6]. As few as 100 CD133$^+$ cells could initiate tumor formation, but 10^5 CD133$^-$ cells could not lead to tumor formation. However, Son et al. found that about 40% freshly isolated human glioblastoma multiforme (GBM) specimens did not contain CD133$^+$ fraction when marked by AC133 antibodies but caused tumors [51]. It was subsequently determined that these tumorigenic cells highly expressed SSEA-1, which were responsible for tumor formation. Furthermore, Lathia et al. provided evidences that integrin α6 was also highly expressed in glioblastoma stem cells. Similar as brain cancer, breast cancer stem cells (BCSCs) were identified and enriched ESA$^+$CD44$^+$CD24$^{-/low}$Lin$^-$ subpopulation, but other fraction of BCSCs could also be found in some patients [5]. In another study, Ginestier et al. showed that BCSCs were identified in ALDH1$^+$ fraction [72]. However, this ALDH1$^+$ fraction exhibited small overlap with ESA$^+$CD44$^+$CD24^{-low}Lin$^-$ fraction in the same tumor. Similarly, minor overlaps among different CSC fractions were also found in some other tumor types [53,58,73]. In our study, we also found that there was slight overlap between CD133$^+$ cells and HCBP-1$^+$ cells enriched in H460 tumor spheres [74].

In addition, there is overwhelming evidence that miRNAs play a critical role in the regulation of CSC functions. Mounting studies suggest that CSCs exhibit distinct miRNA expression profiling compared with non-CSCs [74–76]. According to the results from Liu et al., phenotypically distinct subpopulations of prostate cancer stem cells (PCSCs) had overall distinct miRNA expression patterns, which validated the heterogeneous nature of PCSCs [75]. The miR-188 was overexpressed in the SP of MCF-7, a breast cancer cell line [77], while the upregulation of miR-125b was associated with the SP in breast cancer [78]. Altogether, these results demonstrate that there exists heterogeneity in CSC phenotype among patients or even in the same tumor.

Table 1
Cell surface markers of cancer stem cells.

Tumor type	CSCs surface markers
Acute myeloid leukemia	CD34$^+$CD38$^-$ [4]
Acute lymphoid leukemia	CD34$^+$CD19$^-$ [45], CD34$^+$CD38$^-$ [46]
Breast cancer	CD44$^+$CD24$^-$ [5,47], CD55$^+$ [48], CD61$^+$ [49]
Brain cancer	CD133$^+$ [6,50], CD133$^+$EGFRvIII$^+$ [27], SSEA-1$^+$ [51], integrin α6^{hi} [52]
Colon cancer	CD133$^+$ [7], EpCAM^{hi} CD44$^+$ [53,54], CD66c^{bright} [55]
Prostate cancer	CD44$^+$α$_2$β$_1$^{hi}CD133$^+$ [56,57]
Pancreatic cancer	CD44$^+$CD24$^+$ESA$^+$ [8], CD133$^+$ CXCR4$^+$ [58], FoxM1^{hi} [59]
Head and neck cancer	CD44$^+$ [60,61]
Ovarian cancer	CD44$^+$ CD117$^+$ [9], CD133$^+$ [62]
Lung cancer	CD133$^+$ [10], CD44$^+$ [63], CD166$^+$ [18]
Liver cancer	CD133$^+$ [64],CD90$^+$ [65]
Gastric cancer	CD44$^+$ [66,67]
Melanoma	ABCB5$^+$ [68], CD133$^+$ [69]

Implications of CSC heterogeneity in CSC targeted therapy

The remission of tumor after primary treatment does not mean the total eradication of cancer. Virtually, few surviving CSCs may lead to cancer relapse later. It is widely believed that more effective therapies are needed to eradicate CSCs. Cell quiescence, protective microenvironment, high expression of drug efflux pumps, more efficient DNA repair, miRNA regulation and anti-apoptosis system are potential resistance mechanisms of CSCs [26,35]. It would be productive to develop CSC targeted therapies based on its resistance mechanisms.

To date, most efforts have focused on CSC targeted therapies. Ideally, it should be noted that therapeutic target candidates should not affect normal stem cell natural physiological activities. CSC surface markers and maintenance pathways have been mostly explored in targeting strategies [79,80]. For instance, EGFRvIII, an EGFR variant specifically expressed in glioblastoma stem cells and the tumorigenicity of implanted tumor cells would be reduced if the EGFRvIII$^+$/CD133$^+$ population were pre-eliminated by bispecific antibody [27]. Furthermore, a recent study showed that colon CSCs entered into quiescent G0 phase and were resistant to 5-fluorouracil (5FU) [81]. Tyrosine kinase c-Yes overexpressed in these quiescent CSCs provided a therapeutic target to eliminate quiescent CSCs during 5FU chemotherapy. On the other hand, hypoxic and

perivascular niches also contributed to CSC multidrug resistance phenotype [35]. For example, CSCs increasing in hypoxic niche could decrease their sensitivity to antiangiogenic agents in breast cancer [36]. In another case, hypoxia was reported to activate the insulin-like growth factor 1 receptor (IGF1R) pathway to increase the lung CSC Gefitinib-resistance [82]. Moreover, Saito et al. had reported that AML stem cells locating in the endosteal region of bone marrow had capability of anti-chemotherapy as a result of CSC quiescence [83]. Conversely, chemotherapy eliminated AML stem cells easily *in vivo* when these cells were treated with granulocyte colony-stimulating factor (G-CSF) which could induce cells to enter cell cycle. Thus, combination therapies represent an efficient therapeutic strategy to eradicate CSCs.

As we mention earlier, miRNAs participate in the regulation of CSC functions. This feature provides a window for CSC targeted therapies. For instance, up-regulation of miR-34a could inhibit prostate CSC growth and metastasis through targeting CD44 which is a surface marker of prostate CSCs [84]. miR-34a also correlated with prognosis of gallbladder cancer [64]. Similarly, down-regulation of let-7 and miR-181 would inhibit hepatocellular cancer stem cell chemoresistance and invasion [85]. Thus, miRNA-based therapies represent another potential novel approach to eradicate CSCs. Some natural agents are reported to be the regulator of miRNA expression levels [86]. Sun and colleagues demonstrated that Curcumin (diferuloylmethane) would alter the miRNA expression profiles in pancreatic cancer, which would be a potential inhibitor to pancreatic cancer cell proliferation [86,87]. Moreover, miR-21, whose upregulation was associated with lung tumors, was shown to be inhibited by indole-3-carbinol [88]. Taken together, targeting to CSC-related miRNA markers might be an effective strategy for CSC targeted therapy.

Strong evidence is emerging to support the link between CSCs and therapy resistance (including chemotherapy resistance and radiotherapy resistance) [89,90], which reveals that therapy-resistance would be a promising target for CSC targeted therapy. For example, a potentially effective agent, *Clostridium Perfringens* Enterotoxin, was reported to eradicate the chemotherapy-resistant $CD44^{+}$ human ovarian CSCs [91]. Another report from Awad and colleagues showed that a small-molecular inhibitor named YK-4-279 could decrease the cell viability of chemotherapy-resistant $ALDH^{high}$ stem-like cells in Ewing's sarcoma through inhibiting the EWS-FLI1 oncoprotein [92]. While it is impossible to identify the biomarkers for all CSC subpopulations, targeting to the CSC functional properties, such as the therapy-resistance, could be a new approach to CSC eradication.

Recently, some reports have demonstrated that agents, diagnostic or therapeutic strategies are being developed to target to certain subgroups of CSCs [93,94]. Considering its controllable infection to cells, inherently cytotoxicity to cancer cells and easily genetic engineering [95], oncolytic herpes simplex viruses (oHSVs) are widely studied in metastatic cancer and CSC therapy [96]. Some researches pointed out that oHSVs is regarded as an impactful method to kill breast CSCs, especially the $CD44^{+}CD24^{-/low}$ breast CSCs [44]. Li et al. showed that lapatinib, an EGFR/HER2 pathway inhibitor, selectively eliminated $CD44^{+}CD24^{-}$ breast CSCs [50]. However, lapatinib would not be effective for some other breast cancer subtypes, because of the CSC heterogeneity [59]. Moreover, the increase of $CD44^{+}CD24^{-}$ breast CSCs may be a poor prognostic index for the primary systemic breast cancer therapy. In sum, agents targeting specific CSC subtypes, e.g., $CD44^{+}CD24^{-}$ subtype, have the potential for applying in certain cancer therapy and prognosis [47]. Due to the plasticity and heterogeneity of CSCs and uncertainty of CSC biomarkers, single agent may not be adequate in targeting therapy. As the development of individual therapy in the future, CSC heterogeneity theory could be guidance because the CSC markers or properties in distinct patients are distinctive.

Identification of heterogeneity in CSCs

Although the role of biomarkers on the identification of CSCs was questioned, it is still necessary to find more effective markers for CSC identification and heterogeneity study [11]. Genetics mutation analysis, next generation sequencing, gene expression and proteomic profiling are widely applied in cancer biomarker discovery [97]. As a high-throughput cell surface profiling, flow cytometry combined with antibodies is a commonly used method in marker identification [98]. In addition, given CSC phenotypes are unstable and heterogeneous among patients and markers are highly expected to be applied in early-stage diagnosis and prognosis estimation, CSCs defined by markers should be confirmed by functional assays [24,25]. If the marker candidates have functional significances between CSCs and non-CSCs, it would be more effective to be applied in CSC identification [52]. For instance, Lathia et al. identified integrin α6 as a novel GSC surface marker, which played a key role in GSC self-renewing and proliferation [52]. Recently, anthrax toxin receptor 1 (ANTXR1) was considered as a functional biomarkers in breast cancer stem cells, which was overexpressed in metastatic breast cancer stem cells and related to the poor recurrence-free survival among patients with estrogen receptor (ER)-negative breast cancer [99]. Ai-Fang Huang and colleagues reported that CD164 would promote the ovarian cancer formation through upregulating the SDF-1α/CXCR4 axis [100]. NCAM was reported to be an effective marker for the identification of CSCs from human Wilms' tumor [101]. ANTXR1 and CD164 as CSC markers have been validated by mammosphere assay and animal models, while NCAM has been validated as CSC marker with functional Extreme Limiting Dilution Analysis (ELDA) assay. In summary, CSC surface markers with functional significances would represent an effective therapeutic strategy [52,55].

Even without knowledge of specific CSC markers, some methods have been developed to study the heterogeneity of CSCs such as using specific binding small molecules. In our previous study, specific binding peptides for lung tumor sphere were screened and selected through bacterial surface display method [74], which provided a new strategy for identifying the heterogeneous CSCs *in vitro*, though some functional assay should be carried out to validate the CSC properties in the tumor spheres. Aptamers selected by cell-SELEX strategy were used to identify CSC subgroups successfully in prostate cancer [102]. Subsequently, Kim et al. used the same technique to identify brain cancer stem cells [103]. All these targeting molecules represented novel agents for CSC identification and therapeutic utility. Further investigation using these molecules might contribute to the biomarker discovery in the future. Since CSCs are heterogeneous and their phenotypes are unstable, we can extend these approaches to identify CSCs in a broad range of cancers. Even only one or two subpopulations of CSCs could be targeted and eradicated efficiently, there would be a substantial improvement in cancer therapeutics.

Conclusion

Cancer stem cells are heterogeneous populations and undergo clonal evolution. In addition, diverse microenvironment can also contribute to CSC heterogeneity. Thus, CSC markers are not uniform among patients or even in an individual tumor. Targeting to CSCs depending on the mechanisms such as cell quiescence, protective microenvironments would be effective strategies for targeting therapies to heterogeneous CSCs. Due to the unstable CSC phenotypes, CSCs that are identified by markers should be confirmed functionally. Without the universal surface markers for CSCs, targeting molecules for functionally validated CSCs could be used to identify CSCs and develop novel therapeutic strategies for personalized therapies.

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Conflict of interest

The authors declare no conflict of interest.

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